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J. MICROENCAPSULATION, 1990, VOL. 7, NO. 4, 479–489

Dermal drug delivery by liposome encapsulation: Clinical and electron microscopic studies

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(*Received 27 November 1989; accepted 11 December 1989*)

The fate of liposomes and the encapsulated drug was studied after topical application on the skin. Lidocaine applied on the forearm of human volunteers produced greater local anaesthetic effect in the liposomal form than in the cream form ($p \leqslant 0{\cdot}001$ after 1 h application). Autoradiography demonstrated higher concentration ($p \leqslant 0{\cdot}01$) of ^{14}C-lidocaine in the epidermis and dermis of guinea pigs treated with liposome-encapsulated lidocaine as opposed to lidocaine in Dermabase® cream. Electron microscopic observations, using colloidal iron as an electrondense marker, indicated that intact liposomes penetrated into the skin and deposited in the dermis where they acted as a slow release depot system. On the basis of results in the human volunteers and animals, a hypothetical model for liposome–skin interaction is proposed.

Introduction

In search of improved dermatological products, attempts are being made to design new vehicles or utilize drug carriers to ensure adequate penetration and more importantly, localization of the drug within the skin.

Previous reports from our (Mezei and Gulasekharam 1980, 1982, Mezei 1985, 1988, Gesztes and Mezei 1988) and other laboratories (Krowczynsky and Stozek 1984, Siciliano 1985, Westerhof 1985, Wohlrab *et al.* 1989) indicated the potential of liposomes for dermato-pharmacotherapy. In animal experiments the liposomal form, compared with the conventional dosage forms (ointment, cream, gel, lotion), provided higher drug concentration in the intended site of action i.e. the skin, and lower concentration in the internal organs, i.e. the possible site of adverse or unwanted effects. A mechanism by which liposomal encapsulation achieves the selective drug delivery, higher cutaneous and lower percutaneous absorption, was postulated in a previous report (Mezei 1985). The objective of the present investigation was to test the hypothesis that liposomes penetrate the skin, carry their content into the skin and serve as a depot. The experimental approach was twofold. First, the demonstration of the effect of a drug on the skin in liposome-encapsulated form, but not in a conventional dosage form could confirm that the liposome-encapsulated drug was delivered into the skin in a pharmacologically active concentration. Second, if the presence of intact multilamellar liposomes could be demonstrated within the skin, it would give evidence for the 'depot-formation' hypothesis. Local anaesthetic agents in conventional dosage forms, such as cream or ointment, were shown to lack efficacy on unbroken skin (Dalili and Adriani, 1971). Therefore, we selected lidocaine for liposomal encapsulation and clinical testing.

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The advantage of using a local anaesthetic agent is that the topical anaesthetic effect can be easily assessed by pin-prick method (Evers *et al.* 1985).

To visualize the fate of topically applied liposomes, an electron microscopic technique was employed. A newly developed electrondense marker (colloidal iron) (Foldvari *et al.* 1988) was encapsulated into liposomes which could provide visual evidence for the presence of intact liposomes and liposome-entrapped material within the skin.

Materials and methods

Preparation of liposomes

Encapsulation of lidocaine. Lidocaine was encapsulated into large multilamellar liposomes (MLV), prepared from phosphatidylcholine (Phospholipon 100 from Natterman Phospholipid Gmbh, Cologne, Germany) and cholesterol (Sigma Chemical Company, St Louis, MO) 6·5 : 1 molar ratio (m.r.) by a solvent evaporation method (Mezei and Nugent 1984). Briefly, the lipids, lidocaine and ^{14}C-lidocaine (IZINTA Isotope Trading Enterprise of the Institute of Isotopes, Budapest, Hungary; specific activity 3·15 mCi/mmol) were dissolved in chloroform–methanol 2 : 1 (v/v) and the organic solvent was evaporated under vacuum in the presence of glass beads. The drug : phospholipid molar ratio in the preparation was 1 : 1·43. The lipid film was hydrated at room temperature with a solution containing $NaHCO_3$ 0·65 per cent w/v and NaCl 0·45 per cent w/v. After liposome formation 0·8 per cent w/v hydroxypropyl-methylcellulose was added to increase the viscosity of the preparation. The radioactivity of the preparation was 2 μCi/2 mg drug/0·1 ml. For the human experiments the same formulation was employed except the radioactive lidocaine was excluded.

Encapsulation of colloidal iron. Colloidal iron was encapsulated into MLV prepared from dipalmitoylphosphatidylcholine and cholesterol 2 : 1 m.r. After the evaporation of the organic solvents as described above, the thin, even lipid film deposited on the surface of the beads and the wall of the flask was hydrated with a diluted colloidal iron solution (the stock solution, diluted with 0·01 M PBS 1 : 1 v/v). The liposomes were shaken for 20 min at 45°C (Foldvari *et al.* 1988).

Preparation of control dosage form. Lidocaine was mixed with the appropriate amount of labelled compound and incorporated into Dermabase®, an emulsion-type all purpose ointment base, on a hot water bath (70°C) by mixing, to contain 2 per cent w/w drug. At this temperature lidocaine is melted (m.p. 68–69°C) and can be dissolved in the ointment base. The preparation had been stirred until cooled. The ointment prepared this way was homogeneous; no crystals were observed by light microscopic examination. The radioactivity was the same as in the liposome preparation.

Assessment of the efficacy of liposome-encapsulated lidocaine in human volunteers

Five volunteers (20–25 years of age) have participated in the study following their written consent. In a double-blind design the test (lidocaine liposomes) and control (lidocaine in Dermabase® cream) preparations were randomly numbered and paired by a person not participating in the experiments. A single dose of 0·25 ml of the test or the control preparation was applied on a 10 cm^2 area on either arm of the

volunteers in a random fashion. The treated area was covered with Blenderm® tape (3M Co, St Paul, Minnesota) to provide occlusion for 1 h. The pin-prick test was used to assess the local anaesthetic effect. The device for the pin-prick test consisted of a safety pin opened at 90° angle and pushed through a rubber stopper, which prevents the pin from penetrating the skin (Santos *et al.* 1987). This device has been used by anaesthetists for testing of sensory neural blockade during regional anaesthesia. The advantage of the device over a needle is more uniform stimulus intensity and prevention from skin injury. A new pin was used for each subject, and the length of the pin protruding through the stopper adequate to produce a definite sensation of pin-prick was adjusted for every person. Testing was done immediately after removal of the sample and at 30 min, 1 h, 2 h and 3 h afterwards.

Treatment of guinea pigs with liposomes containing ^{14}C-lidocaine

The hair was removed with an Oster electric clipper (blade No. 40) from the backs of six albino guinea pigs weighing 250–300 g, the day before the experiment. An area of 30 cm^2 was encircled with a marking pen on the shaven back using a template. A volume of 0·6 ml (equivalent to 12 mg [12 μCi] lidocaine) of the preparations delivered from a tuberculin syringe without a needle, was evenly spread on the 30 cm^2 skin area and covered with Blenderm® tape to form an occlusive dressing. One group of animals (three guinea pigs) was treated with a liposome preparation containing 2 per cent lidocaine and the other group (three guinea pigs) with 2 per cent lidocaine incorporated in Dermabase®. After 90 min treatment, the animals were killed under CO_2 atmosphere.

Electron microscopic autoradiography of skin treated with cream or liposomes containing ^{14}C-lidocaine

Small cubes (2 × 2 mm) of the dissected skin samples obtained from guinea pigs treated with cream or liposomes containing ^{14}C-lidocaine were fixed in 2·5 per cent glutaraldehyde in 0·1 M sodium cacodylate buffer pH 7·3 for 2 h at 4°C. After rinsing with the buffer (3 × 10 min), the samples were further fixed with 2 per cent osmium tetroxide in buffer for 2 h at 4°C. The samples were rinsed with distilled water and dehydrated in graded concentration series of acetone, embedded in Taab epoxy resin and cured at 60°C for 48 h. Thin sections of about 70–80 nm thick were cut with a diamond knife on an ultramicrotome. Thin sections were collected on 400 mesh parlodion coated copper grids, coated with carbon and dipped into Ilford L4 photographic emulsion (diluted 7 : 3 v/v). The grids were secured on glass slides for easier handling using double sided sticky tapes. The photographic emulsion coated slides with the sections were dried in the dark and then kept in light proof boxes. Samples were developed in Dektol (1 : 1 v/v) and fixed in Kodak fixer (F-24) 3 weeks later. The sections were poststained with 2 per cent uranyl acetate and 0·5 per cent lead citrate prepared according to Hanaichi *et al.* (1986) and viewed in a Philips-200 electron microscope.

Imaging the topically applied liposomes with colloidal iron as an electrondense marker on electron microscopic level

Four guinea pigs (200–250 g) were shaved on the dorsal area as described earlier. Five 2 × 2 cm areas were marked; one for treatment and four others for controls. On each of the designated areas 0·5 ml of one of the following preparations was applied three times (once every 12 h): (1) colloidal iron loaded liposomes (treatment), (2)

control liposomes (same lipid composition, except no encapsulated colloidal iron), (3) control liposomes mixed with free colloidal iron, (4) free colloidal iron solution. The fifth area served as untreated control skin. Twelve hours after the third topical administration of the above samples, the animals were killed by peritoneal injection of an overdose of pentobarbital. Skin samples from each area were dissected and immediately processed for electron microscopy described in the previous section, except here the samples were treated with 0·25 per cent uranyl acetate overnight after the osmium tetroxide step. Sections were collected on 400 mesh copper grids and post-stained with 2 per cent uranyl acetate. Lead stain was avoided, to prevent the masking effect of lead on the iron particles. One third of the samples were fixed in 2·5 per cent glutaraldehyde in sodium cacodylate buffer pH 7·3 for 2 h at 4°C and in 2 per cent osmium tetroxide for 2 h at 4°C, then dehydrated and embedded as described above. Another part of the samples was fixed in 2·5 per cent glutaraldehyde in 0·1 M sodium cacodylate buffer pH 7·3 for 2 h at 4°C, then dehydrated and embedded into Taab epoxy resin. Heavy metal salts were not used here.

Results

The effect of lidocaine encapsulated into liposomes or incorporated into Dermabase® cream, measured by the painless scores, is shown in table 1. Lidocaine in a liposomal dosage form provided efficient analgesia of the intact skin, which is reflected in the high painless scores. The differences between the cream and liposomal dosage form were statistically significant at every time point, except at the removal of the preparations. The anaesthetic effect produced by the liposome-encapsulated lidocaine was longer than the cream form. Four hours after the removal of the preparations the effect of liposomal lidocaine was still about two times greater than the conventional dosage form.

Evidence for the localization of lidocaine after topical application in liposome or cream form was obtained by autoradiography of thin sections from the excised skin of guinea pigs. The accumulation of drug, represented by the silver grains, in the epidermis and dermis after liposome treatment was found (micrographs not shown). Lower number of silver grains were present in the skin, which was treated with the cream. Statistical analysis of the silver grain density of five randomly selected micrographs from each experiment showed that a significantly higher amount of drug was present in the epidermis and dermis when the lidocaine was applied in liposome-encapsulated form (figure 1).

Table 1. Mean painless scores at different times of observation after 1 h application of 2 per cent lidocaine in liposomes and Dermabase® (control). Number of volunteers = 5. Statistical analysis by paired *t*-tests.

	Liposome		Dermabase®		
Time	Mean	S.D.	Mean	S.D.	*p*
At removal	6·2	3·56	1·8	2·49	0·103
30 min	7·4	3·71	2·6	1·67	0·018
1 h	9·8	0·45	3·6	0·89	<0·001
2 h	8·6	1·14	3·2	1·30	<0·001
3 h	4·6	3·13	2·2	2·17	0·051

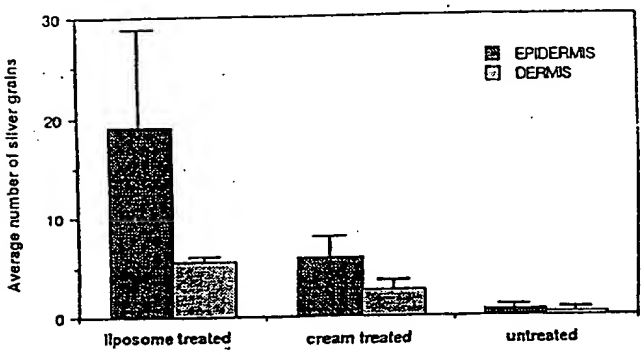

Figure 1. Statistical analysis of the distribution of silver grains in autoradiographic thin sections of guinea pig skin. Guinea pigs were treated topically with ^{14}C-lidocaine incorporated into liposomes or Dermabase® cream, or left untreated, followed by excision of skin and processing for electron microscopic autoradiography as described in Methods. Paired *t*-tests were carried out on the basis of five separate micrographs for each treatment; y = the mean of number of grains ± S.D. Significance level for the distribution of lidocaine in liposome versus cream dosage form: $0{\cdot}05 < p \leqslant 0{\cdot}1$ (epidermis); $0{\cdot}005 < p \leqslant 0{\cdot}01$ (dermis).

To gain insight into the mechanism and sites of drug release from the liposomes and the *in vivo* deposition of lipid vesicles, guinea pigs were treated topically with liposomes containing the electrondense colloidal iron marker and the skin was investigated under the electron microscope.

The electron micrographs showed the presence of intact liposomes in the dermis. The majority of liposomes found in the skin were unilamellar and had a size distribution of 300–500 nm (figure 2 A–D). Multilamellar liposomes could also be found, but less frequently than unilamellar ones (figure 3 A).

The colloidal iron proved to be a suitable marker for liposomes, because it could be encapsulated in high concentration into the liposomes, i.e. into the liposome interior and between the lamellae. The characteristic density of the iron grains in the liposomes represent the intact state of the vesicles, which can be assessed on the micrographs. Figure 2 E shows a typical unilamellar liposome with encapsulated colloidal iron; figure 3 B depicts a colloidal iron containing multilamellar liposome from the original liposome preparation.

We conducted experiments where the skin, treated with liposomes containing colloidal iron, was fixed with 2·5 per cent glutaraldehyde only and no heavy metal (osmium, uranium, lead) salts were used for either fixation of staining. The purpose of this step was to identify clearly the presence of the liposome marker (colloidal iron) in the skin, even though the ultrastructure (re. liposome membranes) was very poor. Colloidal iron particles, arranged in small groups and also scattered iron grains could be found in the sections which were fixed with aldehyde only (figure 4). The location of the marker, as indicated by the presence of collagen fibres, is in the dermis. The small groups of colloidal iron grains could indicate the liposomes, although the liposomal bilayer cannot be identified, since no lipid fixative was used. Figure 5 shows iron particles penetrating partially the stratum corneum. These particles probably derived from ruptured liposomes at the surface of the skin. Other areas of the same section of the colloidal iron liposome-treated skin contained evenly distributed iron grains, similar to that observed with the free colloidal iron solution-treated skin. The route of penetration of the liposome-encapsulated material is

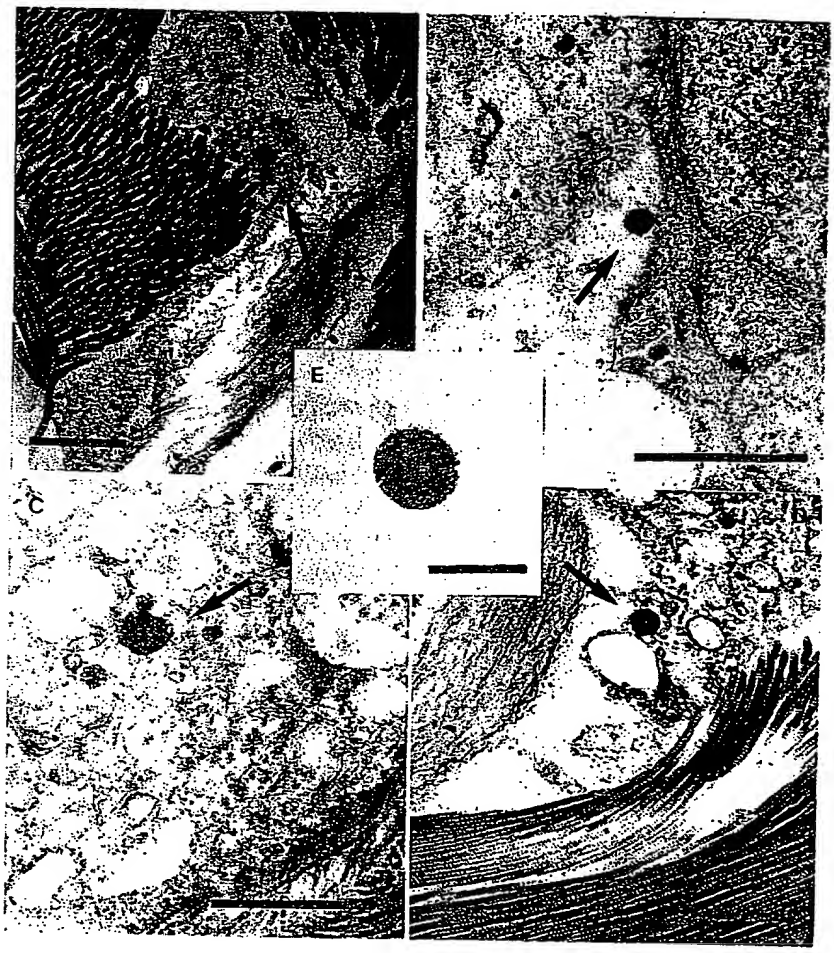

Figure 2. Ultrastructural localization of topically applied liposomes loaded with colloidal iron as a marker. Micrographs A–D show the presence of unilamellar liposomes (arrows) of about 250–300 nm diameter in the dermis. Bars 1 μm. Micrograph E depicts a typical unilamellar liposome loaded with colloidal iron to help the identification of similar structures in the skin. Bar 500 nm.

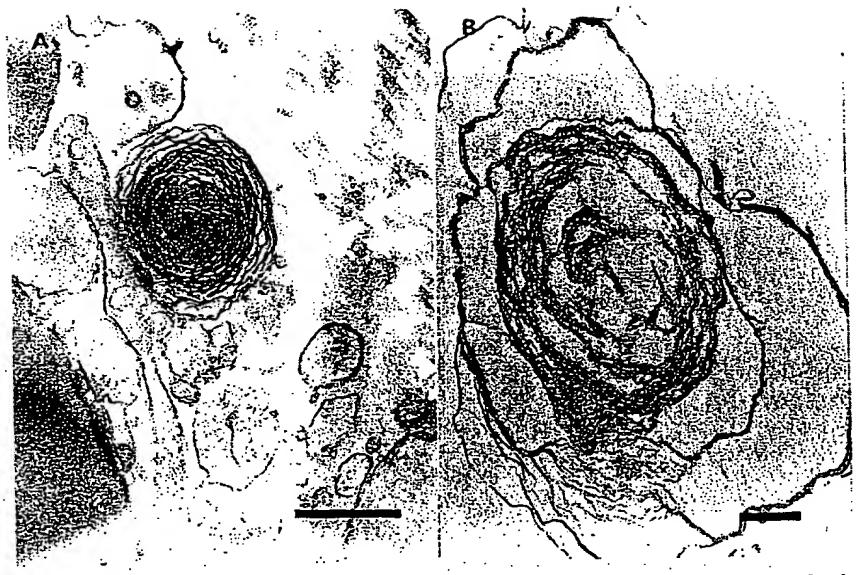

Figure 3. Identification of a multilamellar liposome in the skin. Micrograph A shows a multilamellar liposome localized in the dermis. Micrograph B depicts a typical colloidal iron loaded multilamellar vesicle. Bars 500 nm.

Figure 4. Identification of liposome-encapsulated electrondense material in the skin. After treatment the skin samples were fixed with glutaraldehyde only and no electrondense fixative or stain was applied. Colloidal iron particles arranged in small groups (large arrows) or scattered iron grains (small arrows) can be observed in the dermis area. Bar 500 nm.

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Figure 5. Colloidal iron grains, possibly derived from disrupted liposomes, are partially penetrating the stratum corneum. Bar 500 nm.

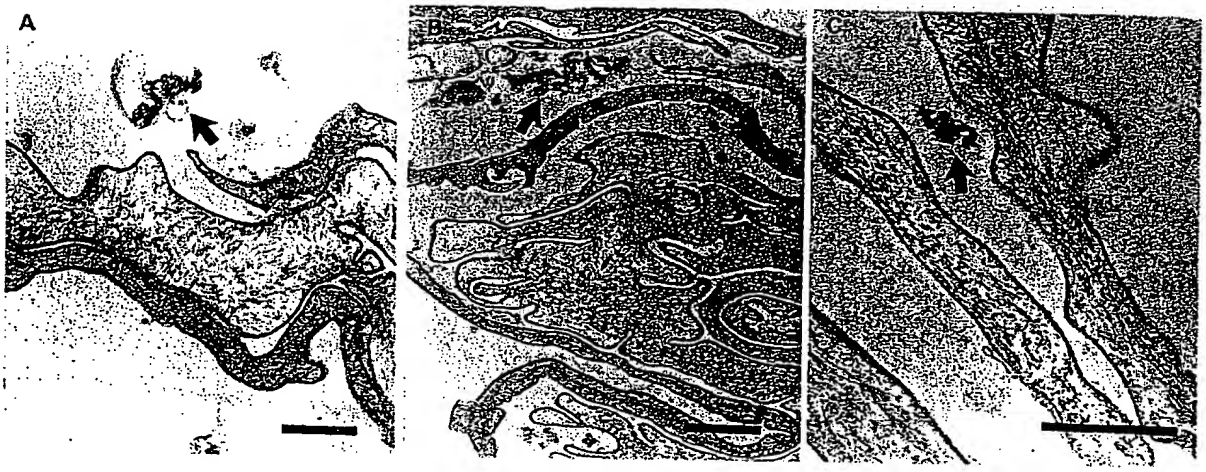

Figure 6. Detection of possible liposomal derivatives within the skin. Micrographs A and B demonstrate colloidal iron particles associated with presumably liposomal lipid material (arrows). Micrograph C shows liposome-encapsulated material penetrating the stratum corneum via the intercellular pathway (arrow). Bars 500 nm.

demonstrated in figure 6 A–C. In figure 6 A and B, the iron particles are still associated with liposomal lipid material, although the liposomal structure is highly distorted. This could be due to the narrow channels in the skin, or partial degradation of the vesicles.

Discussion

One of the most debated areas of skin research is the mode of penetration of different substances into or through the skin. Molecules applied to the skin can penetrate via the intercellular route, appendages or across the bulk of the stratum corneum (intracellular pathways) (Elias 1981, 1985, Barry 1983) depending on their size and polarity. There is no critical size determined so far which would prevent the diffusion through the skin, however, the larger or more polar the molecule the higher the possibility for the shunt route (through the appendages) (Barry 1983). Experiments in human volunteers with liposome-encapsulated lidocaine indicate that liposomes facilitate the penetration of the drug into the skin. The site of action of lidocaine is in the dermis (dermo-epidermal junction) where the peripheral nerves can be found. The demonstrated pharmacological effect (table 1) of lidocaine in liposomal form is the result of the blockade of the sensory nerve conduction. This result implies that the drug must be present in a relatively high concentration in the vicinity of the peripheral nerves in order to produce an anaesthetic effect. The results of the autoradiography experiments also indicate that liposomal encapsulation had an effect on the distribution of silver grains (^{14}C-lidocaine) in the skin. Higher concentration of drug in the epidermis and dermis was found after treatment of the skin with liposomal lidocaine as opposed to treatment with a conventional dosage form (lidocaine incorporated into Dermabase®). These results confirm previous reports by Mezei (1985) and Patel (1985), and moreover provide evidence for the bioavailability of liposome-encapsulated drug. It is of major importance that not only the drug is present in sufficiently high concentration at the site of action, but its properties are not altered in any way (not metabolized or coupled to a vehicle which would prevent its partition into the biological site of action).

The fact that lidocaine produced anaesthetic effect of the skin when applied in liposome-encapsulated form indicates that the liposome vehicle is responsible for the effect, since, with lidocaine in a cream dosage form, only a marginal effect could be demonstrated. The presence of intact liposomes within the dermis signifies that some liposomes can penetrate the skin. It seems, however, that there is a size restriction to penetration, because liposomes larger than about 0·7 μm were not observed.

Both the autoradiography and electrondense marking techniques confirmed the presence of liposome-encapsulated drug within the skin. This could be due to the release of drug from the liposomes at the skin surface and penetration of 'free' drug or the slow release of drug from the liposomes after penetrating into the skin. The simultaneous occurrence of these processes is also possible. 'Liposome-like' material found between corneocytes points to a possible intercellular pathway as penetration route. It is feasible to speculate that smaller liposomes, being flexible lipid vesicles, can penetrate through the 'lipid channels', that is, the lipidic material distributed in the intercellular spaces (Elias 1981, 1985). One may argue that the diameter of the lipid channels within the epidermis is too narrow for the penetration of liposomes of 300–700 nm in size. But one should realize that the diameter of lipid channels were measured after dehydration of the sample for electron microscopic examination, which may not represent the exact *in vivo* diameter. The liposomes are very flexible lipid vesicles, one can filter large size of liposomes, with 5000–10 000 nm diameter through a polycarbonate filter with a pore size of 200–400 nm. Considering this and the dynamic nature of the lipid channels, it is possible that some liposomes can penetrate into the epidermis and dermis. The micrographs (figures 2 and 3) prove this possibility.

The previous studies of Ganesan *et al.* (1984) and Ho *et al.* (1986) found in *in vitro* cell diffusion studies that the liposomal entrapment of hydrocortisone, progesterone and glucose decreased the permeability coefficient of these drugs. Since no phospholipids (of liposome origin) could be detected in the receiver flask, which was separated from the donor flask by excised hairless mouse skin, the above authors concluded that liposomes do not penetrate the skin. This conclusion could be debated. These *in vitro* studies (Ganesan *et al.* 1984, Ho *et al.* 1986) measured only percutaneous (through the skin) and not cutaneous (into the skin) penetration. Since the presence of liposomes within the skin samples was not analysed, the conclusion could be valid only for transdermal, rather than dermal penetration.

Another possible way for the 'penetration enhancer' effect of liposomes is that the major component of liposomes, phospholipids, can provide an aid to increase the continuity of the phospholipid matrix of the skin, which can facilitate the movement of lipophilic molecules (Keith and Snipes, 1982). This latter speculation can explain why liposome encapsulation appears to promote the penetration of hydrophobic substances more than hydrophilic compounds (Mezei 1985, Ganesan *et al.* 1984), but it cannot shed light on how the liposomes can maintain a long duration of action of the encapsulated drug. On the basis of the present experimental evidence we propose that liposomes can penetrate the epidermis, and carry the drug into the skin. The multilamellar structure of liposomes protects the encapsulated drug from premature clearance (metabolism and uptake into the blood circulation), and in addition can provide a vehicle for sustained release of drugs. It is reasonable to speculate, however, that in the liposome–skin interaction process more than one event takes place. These events are summarized in figure 7. Multi- and unilamellar

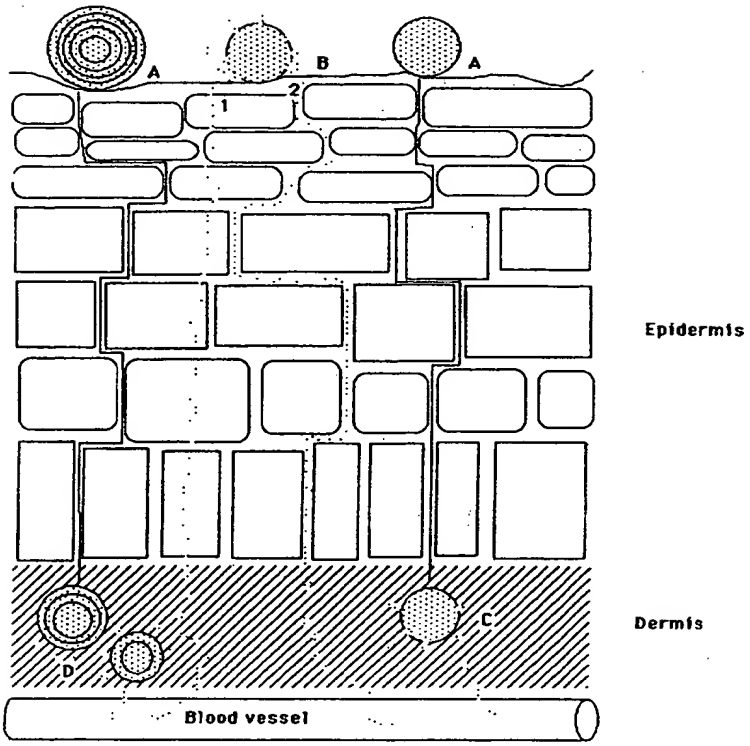

Figure 7. Proposed mechanisms for the interaction of liposomes with the skin. A, Adsorption of liposomes to the skin surface; drug transfer from liposomes to skin. B, Rupture of vesicles, release of content and the penetration of the free molecules into the skin via 1 = intracellular, 2 = intercellular route. C, Penetration of unilamellar vesicles via the lipid-rich channels to the dermis where they slowly release their content due to disruption or degradation of liposomal membranes. D, Penetration of multilamellar vesicles via the lipid-rich channels. On the route of penetration of multilamellar vesicle can lose one or more outer lipid lamellae which would lead to partial release of the encapsulated material.

liposomes can be absorbed to the skin surface intact (figure 7 A) before their penetration into the skin. Some liposomes can rupture on the surface of the skin (figure 7 B). The penetration of smaller vesicles is more probable (figure 7 C), however, it is possible that the intradermally localized uni- or oligolamellar vesicles are derived from multilamellar liposomes, which lost their outer bilayers during penetration (figure 7 D).

Conclusion

Liposomes are prime candidates as vehicles for the topical delivery of drugs. Liposomes can carry the encapsulated drugs into the skin and provide a sustained release. The complete reconstruction of events in the liposome–skin interaction process, however, will require further studies.

Acknowledgements

We wish to thank Dr Gary T. Faulkner for the use of EM facilities and the useful discussions. The excellent technical assistance of Beverly Greenlaw is greatly appreciated. This work is supported by MRC (Canada) Grant No. MT6664.

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